CHARACTERIZATION OF ENDOGENOUS APP PROCESSING IN A CELL-FREE SYSTEM

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ABSTRACT

We have developed a simple *in vitro* assay using tissue homogenates that allows detection and characterization of several endogenous proteolytic activities which convert Alzheimer's amyloid precursor protein (APP) to the smaller, carboxy-terminal fragments, postulated to be intermediates in the formation of β-amyloid peptide (Aβ). Incubation at 37° C results in the degradation of transmembrane APP and formation of a mixture of carboxy-terminal containing peptides with mass values of 9-12 kDa. Epitope mapping and electrophoretic comparison with a truncated APP standard showed one of these peptides to contain the entire Aβ sequence. Analysis of pH dependence shows that formation of this carboxy-terminal product as well as another fragment, that is the likely product of 'secretase' activity, requires acidic pH. This suggests that cleavage of full-length APP to secreted forms may take place in an acidic intracellular compartment.

INTRODUCTION

Definitive diagnosis of Alzheimer's disease (AD) depends upon the finding of senile plaques within the brain parenchyma and amyloid deposits around the cerebral vessel (CV) walls upon autopsy (1). The major protein component of the core of the senile plaques as well as the CV deposits is a 40-43 amino acid (aa) polypeptide called β-amyloid peptide (Aβ; 2,3). Aβ is derived from a larger gene product, the Alzheimer's amyloid precursor protein (APP), a transmembrane protein found on the cell surface (4). Deposition of Aβ in amyloid plaques (5) and the finding that mutations in APP are inherited by affected individuals in familial AD (6) strongly implicates the involvement of APP in the etiology of the disease.

It has been suggested that Aβ or amyloid plaques are toxic to neurons in culture (7) and in the brain (8), but this finding is still controversial (9,10). Recent reports indicate that production and release of Aβ is a normal event both for cells in culture and brain (11). Therefore it is unlikely that Aβ is toxic in its predominant form. Aβ toxicity may depend on the manner or location of its production or require aggregation (9), fibril formation or deposition into plaques (12). APP exists either as a full-length, transmembrane protein on the cell surface or a truncated soluble extracellular protein, protease nexin 2 (PN2). APP has been postulated to play a role in cell survival (13), neurite outgrowth (14,15), or to act as a cellular receptor (16).

APP is proteolytically processed to form Aβ, which is released into cerebrospinal fluid (11,17) or the media of cells in culture (18,19) in one constitutive APP processing pathway. Another pathway results in release of the large extracellular domain of APP via cleavage near the membrane at a locus within the Aβ sequence, precluding the formation of full-length Aβ (20,21). The processing pathway of APP to Aβ is incompletely understood both with respect to the enzymes involved and the temporal and spatial progression from a transmembrane holoprotein to an extracellular fragment that can form amyloid.

One model for the pathogenesis of AD is that aberrant or overabundant processing results in cleavage at sites within APP that promote the generation of amyloidogenic fragments. Such potentially amyloidogenic fragments, which contain all or part of Aβ, have been isolated from cell culture, whole brain (22) and cerebral microvessels (23). In contrast, 'normal' processing at the α-secretase site within the Aβ sequence precludes the *subsequent* formation of full-length Aβ and is therefore a "non-amyloidogenic" pathway. The identity of the proteases involved in the formation of amyloidogenic fragments or subsequent further degradation to Aβ is unknown. Our goal is to identify the activities that cleave in or around the Aβ region and form carboxy-terminal (C-terminal) fragments of APP (22) that may be intermediates in the

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formation of Aβ (i.e., amyloidogenic) or alternatively cleave within the Aβ region and thereby preclude formation of Aβ (non-amyloidogenic). Modulation of such activities may interrupt the progression of AD by preventing the inappropriate generation of Aβ.

In a previous communication we reported experiments that tested proteases that have been proposed as candidates for the β-secretase, a protease that generates the N-terminus of Aβ, to generate C-terminal fragments from APP (24). Those studies used homogenates of brain tissue or cultured cells as substrates and purified protease preparations. In this report, we have developed a cell-free system that generates C-terminal fragments of APP from native, full-length, membrane-embedded APP, without addition of exogenous proteases. Mammalian brain tissue has been used as the endogenous source of both substrate and proteolytic activity. Using this system we have evaluated the optimal conditions required to detect activities that generate both non-amyloidogenic and potentially amyloidogenic products of APP proteolysis. We find that all of the C-terminal generating proteases require (slightly) acidic pH for activity, although the individual protease pH profiles vary.

METHODS

Reagents

Frozen rat and rabbit brains were obtained from PelFreez. Aprotinin, leupeptin and phenylmethylsulfonylfloride (PMSF) were from Sigma. Protein A-sepharose was from Pharmacia. Antisera 57 (raised against amino acids 748-770 of APP 770) and 3 (positions 724-747 of APP 770) were obtained as previously described (25). The specificity of antisera 57 to APP was investigated by testing the ability of synthetic peptides to prevent binding to ELISA plates coated with the antigenic peptide. Antiserum was pre-incubated with synthetic peptides whose sequence corresponded to the last seven aa of either the APP or Amyloid Precursor-Like Protein (APLP) sequence (26). We found that the APP-derived sequence was at least 80 times more effective as a competitor for binding than the APLP-derived sequence. Similarly, the peptide with APP-derived sequence was able to completely block binding of antisera 57 in western blotting, while addition of peptide with the APLP-derived sequence at the same peptide concentration resulted in normal staining of both full-length APP and C-terminal fragments. In addition, we found that constructs in which amino acids were added to the carboxy-terminal sequence of APP resulted in the loss of immunoreactivity with antisera 57, supporting the conclusion that antiserum reacts specifically with the far C-terminus of APP (J.S. Jacobsen, unpublished). Monoclonal antibodies 4G8 (anti-Aβ 17-24; 27), 6E10 (Aβ 8-12; 20) and 1G6 (APP 573-596; K.S. Kim, personal communication) were provided by K.S. Kim and H.M. Wisniewski (Institute for Biomedical Research, Staten Island, NY). Alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Biorad. Immobilon was from Millipore.

Autolysis of Brain Homogenates

Rat brains were homogenized at 4°C with an Omni 2000 tissue homogenizer (Omni International, Gainesville, VA) in 5 volumes of 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5, unless otherwise noted, and incubated at 37°C. Following incubation for 0 to 4 hr, protease inhibitors were added (1 mM PMSF, 1 mg/ml aprotinin, 10 mg/ml leupeptin), the membrane fraction was collected by centrifugation at 15000 g for 10 min, followed by extraction of the pellet with 1% triton X-100 and 1% deoxycholate, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5. Insoluble material was removed by ultra-centrifugation at 140,000 g for 15 min and the extracted APP was immunoprecipitated overnight using antisera 57 to the carboxy-terminus (C-terminus) of APP and protein A-sepharose. Protein A-sepharose pellets were washed once with phosphate buffered saline, 0.1% SDS, boiled in SDS gel loading buffer containing dithiothreitol, resolved on 4-20% polyacrylamide gels using a tris-tricine buffer system (28) and transferred to immobilon. APP and C-terminal fragments were stained by antisera 57 against the C-terminus of APP at a dilution of 1:100,000 and detected by alkaline phosphatase conjugated goat-anti-rabbit antibody and NBT/BCIP used as directed by the manufacturer. Since the objective of the experiment was to determine the yield of APP fragments after various digestion protocols total protein loading was not equalized across gel lanes. Instead the input of APP-containing homogenate was distributed equally before digestion was initiated.

Analysis of pH Dependence

Tissue was homogenized in 5 volumes of 20 mM Tris-HCl, pH 7.4. Aliquots of homogenate were then mixed with equal volumes of 100 mM glycine-HCl, pH 3; 100 mM Na-acetate, pH 4; 100 mM Na-MES, pH 5 or 6; 100 mM tris-HCl, pH 7 or 8; 100 mM Na-borate, pH 9. The final pH of the homogenate was confirmed and adjusted as necessary.

Cultured Cell Lines

All cells were obtained from American Type Culture Collection and maintained in Delbecco's Modified Eagles Medium (DMEM) and 10% fetal calf serum. They include human glioblastoma/astrocytoma, U-87 MG (ATCC HTB14); SV40-transformed African Green monkey kidney, COS-1 cells (CRL 1650); Adeno-5-transformed human embryonic kidney, 293 cells (CRL 1573); human medulloblastoma, TE671 cells (CRL 8805). Cells were cultured as described (19).

Recombinant Constructs

Construction of C_{t102}, transfection and stable selection of expression in 293 cells was previously described (19). As depicted in Fig. 1a, expression of this construct

a

b
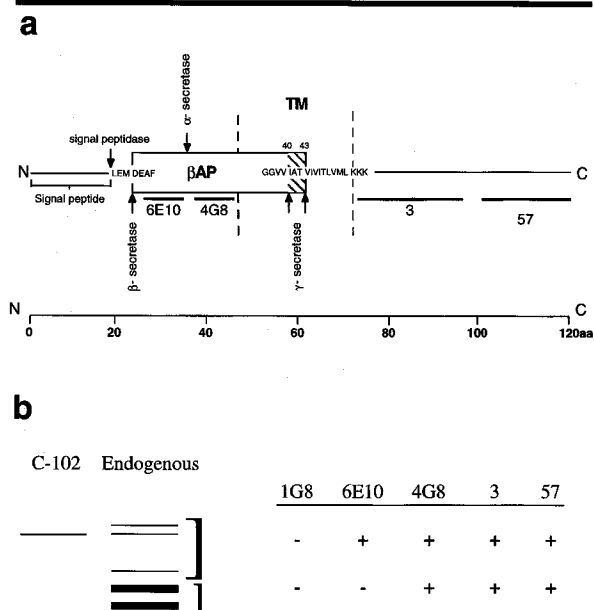
Figure 1: Schematic of APP and C_{1102}. a) Epitope map of antibodies used in this study and diagram of C_{1102} peptide. b) Diagram of immunoreactivity of C-terminal fragments with antibodies to different regions of APP, as indicated in *Method* section.

generates a 120 aa polypeptide containing the 18 aa native APP signal peptide sequence (29). Radiosequence analysis of the overproduced fragment confirms that the signal peptide is cleaved by signal peptidase at the same position as in APP (25), resulting in a 102 aa transmembrane polypeptide (Brown et al, unpublished). The N-terminus of this 'mature' product begins 3 aa prior to Aβ (i.e., LEMDAEF where D is the first aa of the Aβ sequence) and contains the remaining transmembrane domain and cytoplasmic sequences of APP. Expression of this construct also results in increased release of a Aβ-like peptide into the extracellular media. We have previously confirmed by radiosequence analysis of the overproduced Aβ-like fragment that it contains 3 additional amino acids on its N-terminal end (19), which is consistent with the radiosequence result for C_{1102}.

RESULTS

Identification of Brain Membrane-Derived APP and C-Terminal Fragments of APP

Full-length, membrane-bound APP was isolated by detergent extraction followed by immunoprecipitation with a C-terminal reactive antibody that binds to APP but not APLP (20). The identity of the immunoprecipitated proteins was analyzed by SDS polyacrylamide electrophoresis, followed by immunoblotting with a panel of antibodies to different epitopes within APP (see Fig. 1). The full-length APP protein isolated from rat brain appears as a 100-110 kDa doublet (Fig. 2, lane 1), while rabbit brain APP displays an additional ~120 kDa form

(Fig. 2, lane 2). Immunoprecipitation of detergent extracts of membranes from either rat or rabbit brains with anti-C-terminal antibody yields, in addition to the full-length forms, a group of 5 bands with apparent molecular mass values in the 9-12 kDa range (22).
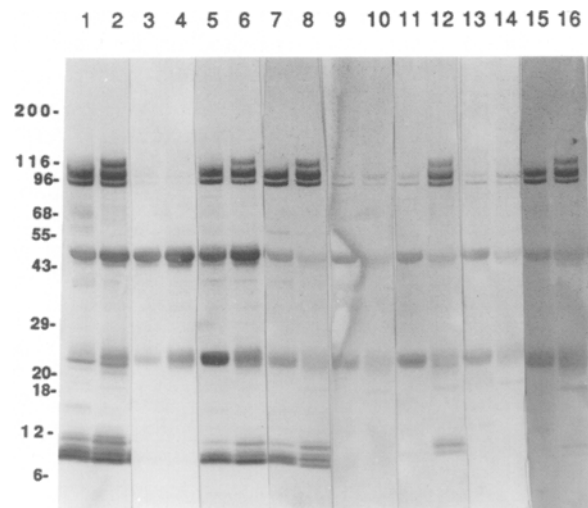
Figure 2: Immunoprecipitated APP fragments from rat brain. Homogenized rat or rabbit brain membranes (100 mg tissue) were extracted with detergent and immunoprecipitated with anti-C-terminal antibodies, resolved by gel electrophoresis and western blotted with antibodies to different APP epitopes as described in *Method* section. Lanes *1* & *2*, antibody 57; lanes *3* & *4*, 57 and competing peptide (APP 748-770); lanes *5* & *6*, antibody 3; Lanes *7* & *8*, 4G8; lanes *9* & *10*, 4G8 and competing peptide (Aβ 17-28); lanes *11* & *12*, 6E10; lanes 13 & *14*, 6E10 and competing peptide (Aβ 1-12); lanes *15* & *16*, 1G6. Odd numbered lanes are from rat, even from rabbit. Note that prominent bands of ~48 and ~24 kDa, in this and all subsequent figures, are due to IgG heavy and light chains, respectively, which were introduced during immunoprecipitation. The faint low molecular weight bands in lane 16 do not correspond in mobility to the immunoreactive bands in lane 12; furthermore, there are no corresponding bands in lane 15 as would be expected since 1G6 reacts equally well with rat and rabbit derived APP.
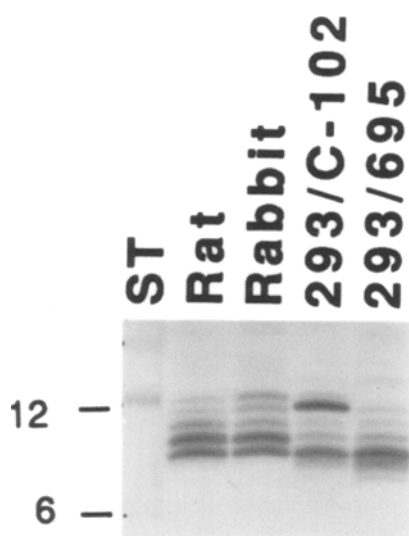
As seen in Fig. 2, only APP or APP fragments from rabbit brain react with antibody 6E10 (lane 11 vs. 12). We attribute the selectivity of 6E10 to the primary sequence difference between rat and rabbit (30) within the epitope recognized by the antibody. The epitope recognized by 6E10 encompasses the region of amino acids 8-12 in the human Aβ sequence (20; shown schematically in Fig. 1a). Rabbit and human Aβ sequences are identical while rat Aβ differs at aa positions 5, 10 and 13 (30). All five C-terminal APP fragments react with antibody 4G8 (Fig. 2, lanes 7 and 8), but antibody 6E10 reacts only with the three larger C-terminal fragments from rabbit brain (lane 12). As summarized in Fig. 1b, the 9 and 9.7 kDa fragments contain the portion of Aβ located C-terminal to the 'secretase' cleavage site, which is between Aβ aa 16 and 17, since they both contain the $A\beta_{17-24}$ epitope recognized by 4G8. Since the three longer fragments

(10.9, 11.4 and 11.8 kDa; 22) react with monoclonal antibody 6E10 they include Aβ sequence that is N-terminal to the α-secretase site. Therefore, the three longer fragments are potential intermediates in the formation of Aβ.
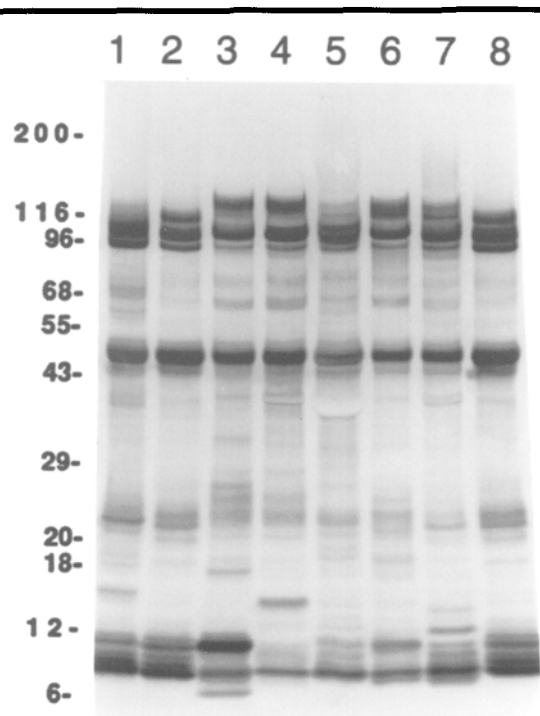
In order to determine which of the three larger C-terminal fragments is likely to contain the entire Aβ sequence, we compared the electrophoretic mobility of these fragments and a truncated form of APP, C_{t102}, which is a C-terminal fragment that contains only 3 additional aa preceding the Aβ sequence. As shown in Fig. 3, stable expression of C_{t102} in 293 cells resulted in overproduction of an 11.4 kDa fragment that is immuno-precipitated and recognized on immunoblots by antise-rum 57. The similar mobilities of the recombinantly expressed and endogenous C-terminal fragments (Fig. 3) indicate that the 11.4 kDa fragment starts near the N-terminus of Aβ. The slightly retarded mobility of the C_{t102} fragment relative to the 11.4 kDa fragment from rabbit or 293/695 cells (Fig. 3) is as expected if the 11.4 kDa begins at the canonical Aβ N-terminus thus forming a shorter C-99 fragment.

Comparison of APP and C-terminal fragments from different Sources

The ~9 kDa brain fragment co-migrates with the major endogenous C-terminal fragment from four different tumor cell lineages, including 293 cells (Fig. 4, lanes 4-7). The original sequence data for the location of the α-secretase cleavage site was derived by sequencing of C-terminal fragments isolated from 293 cells stably


Figure 4: Comparison of APP and C-terminal fragments from different sources. Membrane homogenates from tissue (100 mg wet weight) and cell culture were detergent extracted, immunoprecipitated and electrophoretically resolved, on 4-20% tricine gel, and immunoblotted with antisera 57 as described. a) Lane 1, rat brain; lane 2, rabbit brain; lane 3, EC 293 cells stably expressing C_{t102}; lane 4, 293 wild type cells; lane 5, HTB 14; lane 6, TE 671; lane 7, Cos 1; lane 8, rabbit brain. We sometimes observe an additional ~6 kDa band in extracts of C_{t102} stable cells (cf. lane 3 with lane 3 of Fig. 3).

Figure 3: Comparison of brain-derived C-terminal fragments with C_{t102}. Membrane homogenates from brain tissue (100 mg wet tissue) and cell culture were detergent extracted, immunoprecipitated and electrophoretically resolved, on 16% tricine gel, and immunoblotted with antisera 57 as described. Lane 1, molecular weight standards (kDa); lane 2, rat brain; lane 3, rabbit brain; lane 4, EC 293 cells stably expressing C_{t102}; lane 5, 293 cells overexpressing APP$_{695}$.

overproducing APP (31). The ~9 kDa fragment from brain is the best candidate for the α-secretase cleavage product because it co-migrates with the 293-derived fragment. As summarized in Fig 1b, the ~9 kDa fragment does not react with 6E10, which recognizes Aβ$_{8-12}$, and is the shortest C-terminal fragment that reacts with 4G8, which recognizes an epitope (Aβ$_{17-24}$). Therefore we conclude that it represents the product of α-secretase cleavage.

Based on the assignment of the 11.4 kDa fragment as C-99, only the 11.4 and 11.8 kDa fragments contain intact Aβ and could thus be intermediates in the formation of Aβ (*i.e.*, amyloidogenic). It is worth noting that there are quantitative differences in the relative abundance of the five C-terminal fragments extracted from rat and rabbit brains (Fig. 3, lanes 1 & 2). As seen in Fig. 4, there are even more striking, quantitative and quali-tative differences among different cell lines (lanes 4-7) and between cell lines and brain fragments (cf. lanes 1,2,8 vs. lanes 4-7). For example, TE671, a CNS-derived cell line produces abundant 11.4 kDa fragment.
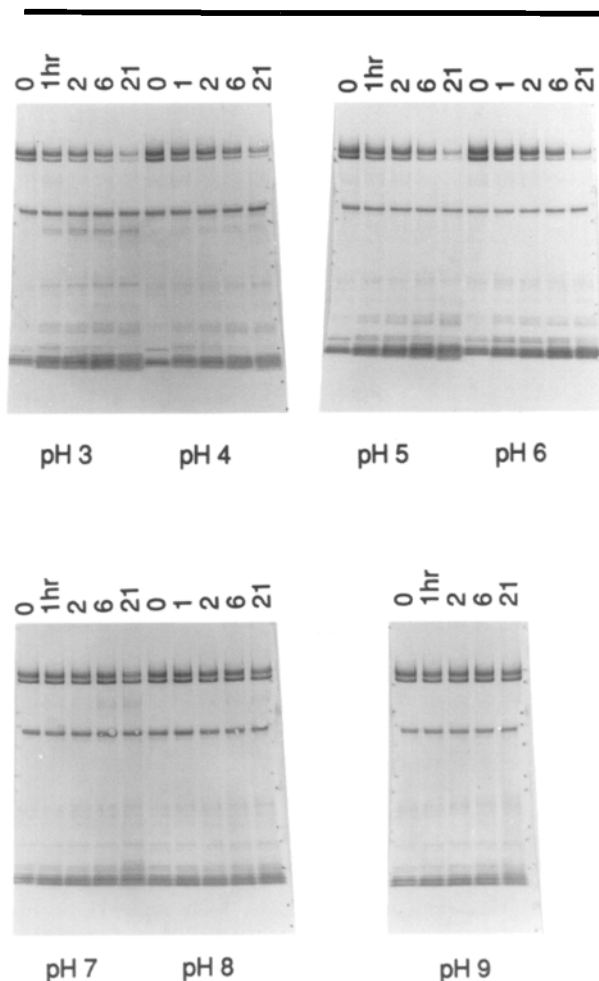
Figure 5: pH dependence of endogenous proteases. Rat brain was homogenized at pH 7.4 and then adjusted to the indicated pH by addition of an equal volume of 10x buffer. Samples were incubated for the indicated times and processed as described in **METHOD** section.

In order to investigate whether some endogenous APP degrading proteases are membrane associated, the membrane fraction was collected by centrifugation of homogenates at 15000 g for 10 min and then washed with 0.5 M NaCl, 10 mM EDTA, 10 mM EGTA, 0.1% triton X-100, or 0.2% octyl glucoside, agents that are known to remove loosely bound membrane-associated components (32). After a second centrifugation step, the washed samples were resuspended and incubated at pH 5. None of the above treatments resulted in changes in the pattern or amount of C-terminal products generated (data not shown). The inability of mild washing to remove the proteolytic activities that generate the 9 kDa fragment suggests that the proteolytic activity may be membrane bound.

Endogenous Degradation of APP Requires Acidic pH

The pH effect reported above could reflect a genuine requirement for enzymatic activity or could be an homogenization artifact that indirectly influences the activity.

These possibilities were distinguished experimentally by homogenization at one pH followed by incubation at another pH. Fig. 6b shows that homogenization at pH 5 followed by incubation at pH 7.4 results in no change in C-terminal fragments or loss of APP (lane 1 vs. 4). In contrast, homogenization at pH 7.4 followed by incubation at pH 5 results in increased C-terminal fragments and loss of APP (Fig. 6f; lane 1 vs. 4). Thus, *in vitro* formation of C-terminal fragments and degradation of APP requires incubation at pH 5 regardless of the pH during homogenization.
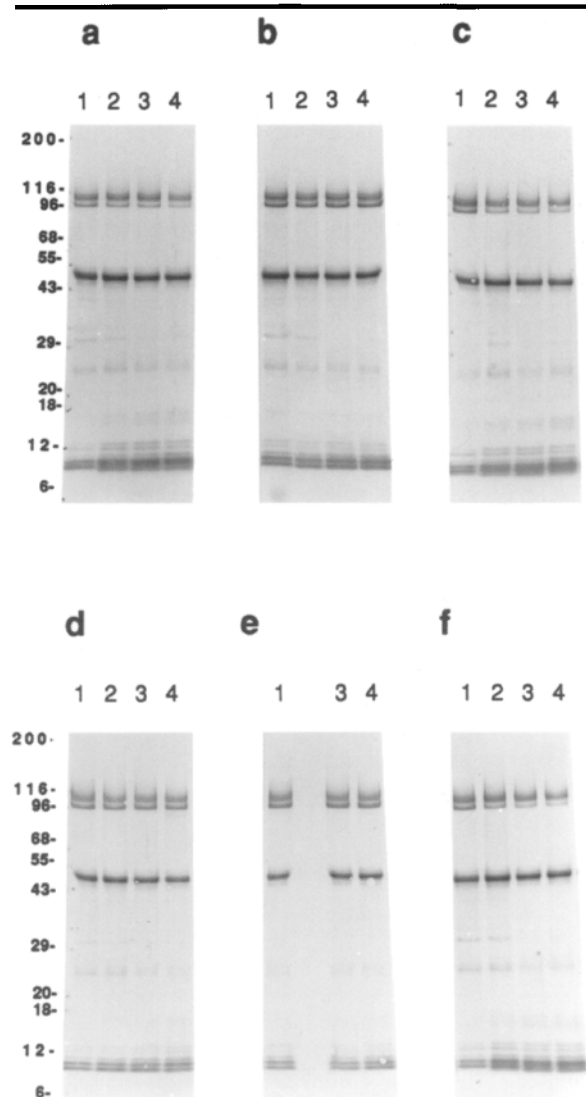
Figure 6: Requirement of low pH incubation for formation of C-terminal fragments. Rat brain was homogenized at initial pH as indicated and then pH was changed by addition of equal volume of 5x buffer and confirmed by direct pH measurement and incubated for 1 hr at 37° C. a) pH 5, b) pH 5 adjusted to pH 7.4 by addition of pH 7.4 buffer concentrate, c) pH 5 with added pH 5 buffer concentrate, d) pH 7.4, e) pH 7.4 with added pH 7.4 buffer concentrate, f) pH 7.4 adjusted to pH 5 by buffer addition. Lanes *1-4* represent times of 0, 1, 2, 4 hours, respectively. Homogenization of brain tissue was done in 5 volumes of 20 mM buffer at indicated pH followed by addition of an equal volume of 100 mM buffer.

DISCUSSION

Various putative APP proteases have been characterized, usually based on their activity in hydrolyzing synthetic substrates or short peptides (24,33-37). In some cases enriched or purified fractions have been tested and found to degrade APP (35) or form C-terminal fragments of approximately the correct molecular mass (25,38). However, the ability of an isolated enzyme to cleave at a particular sequence within the target protein does not prove that it is the activity that actually operates *in vivo*. We have therefore chosen to use a cell-free system in order to address the issue of co-localization of enzyme and substrate and provide comparative information about candidate APP processing proteases.

Selection of a Source of Endogenous APP Proteolytic Activities

We first needed to select an appropriate source that contains both APP and potentially amyloidogenic products of APP. Therefore, we isolated APP and C-terminal fragments of APP from rat and rabbit brains and cultured cell lines by immunoprecipitation with a C-terminal reactive antibody that we have found to bind to APP but not to APLP (26). The fragments thus isolated were identified by epitope mapping on immunoblots using antibodies that recognize different sequences within APP, including monoclonal antibodies (4G8, 6E10 and 1G6) that recognize epitopes that are unique to APP (26,27,39, K.S. Kim, personal communication). Based upon epitope mapping and comparison with C-terminal fragments from previously characterized cell lines (31), we have tentatively identified the ~9 kDa C-terminal band from brain tissue as the fragment generated by the α-secretase cleavage. Also, comparison with the electrophoretic mobility of an APP fragment containing the last 102 amino acids of APP, C_{t102}, overexpressed in 293 cells (19) leads us to identify the ~11.4 kDa endogenous fragment as the best candidate for a C-99 fragment generated by β-secretase beginning with canonical Aβ N-terminal sequence DEAFRH. Radiosequencing of the 11.4 kDa fragment isolated from M17 cells overproducing APP-695 is consistent with an N-terminal sequence of DAEF (40), which further supports our identification.

Differences between APP derived from different sources are evident upon examination of Figs. 2 & 4. Full-length APP isolated from rabbit brain displays an additional abundant C-terminal immunoreactive band with apparent molecular mass of ~116 kDa. Western blotting with antisera Kunitz protease inhibitor (KPI) domain of APP indicates that this extra band is the KPI-containing isoform of APP. RNA for the KPI-containing isoform of APP is essentially absent in rat brain, unlike other species (41) such as rabbit. We have also observed that the relative abundance of the putative C-99 endogenous fragment is greater in rabbit-derived C-terminal fragments than those from rat (Fig. 3). Preliminary results indicate that rabbit homogenate, under pH

conditions similar to those reported here, provides a larger increase in the C-99 fragment than rat homogenate.

Even more pronounced differences are found between brain-derived and culture-derived C-terminal fragments and among different cell lines (Fig. 4, lanes 1-2 vs. lanes 4-7). For example, the 11.8 kDa fragment is generally not detected in the cell lines examined whereas the abundance of the 11.4 kDa fragment varies widely between lines and is generally lower than that found in brain samples. We also observed that the cell lines tested have lower starting levels of C-99 (Fig. 4, lanes 4-7) and increases in C-99 are harder to detect (not shown). The abundance of C-99 found in tissue or cells may be related to the activity of endogenous converting protease, the APP isotype available as substrate, or to other factors such as the rate at which C-terminal fragments are degraded. Although we have not examined whether there is a correlation between the endogenous levels of the 11.4 kDa fragment in different untransfected cell lines and the rate of Aβ release into the conditioned media, overexpression of C-99 constructs does result in increased release of Aβ (19,41). This observation suggests that the greater abundance of C-99 fragment found in brain could result in higher production of Aβ in brain than in culture.

Proteolytic Activities Require Acid pH and are Membrane Associated

The observation that endogenous APP degradation took place when brain tissue was homogenized and incubated at pH 5, but not pH 7.4, may reflect either a requirement of homogenization or incubation. Low pH during the homogenization could result in different shear-induced fragmentation or fusion of cellular membranes that does not take place during homogenization at high pH. Alternatively, low pH is simply a requirement for proteolytic activity. This ambiguity was resolved by homogenization at either high or low pH followed by incubation at the same or other pH. The result of this experiment (Fig. 5) clearly showed that the low pH is required only during the incubation and therefore reflects a property of the proteases associated with generation of the C-terminal fragments.

The putative cleavage by α- and β-secretases proceeds at low pH and is abolished above pH 7 (Fig. 6). This finding supports a model in which soluble APP, i.e. the secreted N-terminal APP fragment (PN2), is formed in an acidic internal compartment rather than on the plasma membrane which is ordinarily exposed to higher pH. Our finding of an acidic requirement for proteolytic activity, particularly the secretase-like activity, also suggests that the mode of action of lysosomotrophic agents in inhibiting APP secretion (43) in tissue culture may be via protease inhibition, rather than by interference with membrane trafficking. That is, lysosomotropic agents inhibit APP release by raising the pH of intracellular compartments, resulting in inhibition of secretase. The model of intracellular formation of PN2 (43,44) is in

contrast to the plasma membrane cleavage model (45). Our unpublished observations suggest that inhibition of the putative secretase activity by protease inhibitors is difficult to detect while manipulation of pH readily alters cleavage activity. This is consistent with the intracellular model, since that model would also predict that secretase activity is associated with the luminal face of organelles, which would not be readily accessed by membrane impermeable reagents, even after homogenization. The interior of these compartments would not, however, be able to regulate pH gradients under the conditions employed, since energy metabolism is not supported.

A study of cleavage of an APP fragment overexpressed in H4 cells in culture has provided evidence for cleavage of membrane APP by a DTT-inhibited protease (46). Some evidence is provided that the α-secretase activity, which cleaves in the middle of the Aβ sequence, may be membrane associated.

In this communication we have demonstrated the ability to analyze APP and the products of APP proteolytic processing from tissue homogenates. We have exploited this ability to detect the formation of C-terminal fragments of APP corresponding to the complement of the secreted form of APP, also referred to as protease nexin 2 or PN2. We also observed the formation of longer fragments that contain the entire Aβ sequence and have identified one which is likely to have the same N-terminus as Aβ. Studies of the pH dependence, as well as fractionation and washing experiments have provided preliminary information about the nature of the protease that generates soluble APP which has been referred to as 'secretase'. Further characterization of the activities that generate these fragments should provide valuable information about the properties, such as inhibitory profile, and localization of the proteases that form Aβ.

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ABBREVIATIONS

The abbreviations used are: aa, amino acid(s); AD, Alzheimer's Disease; APLP, Amyloid precursor-like protein; APP, Amyloid precursor protein; Aβ, β-amyloid peptide; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; C-terminal, carboxy-terminal; C-terminus, carboxy-terminus; CV, cerebral vessels; DTT, dithiothreitol; EDTA, ethylenediaminetetraaceticacid disodium salt; IgG, immunoglobulin G; KPI, kunitz-type protease inhibitor; LEMDAEFHR, NH_2-leucine-glutamine-methionine-aspartate-alanine-glutamine-phenylalanine-histidine-arginine-COOH; MES, 2-(N-morpholino)ethanesulfonic acid; NBT, nitroblue tetrazolium; N-terminal, amino-terminal; PN2, protease nexin 2; PMSF, phenylmethylsulfonylfloride. Amino acid code: A, alanine; D, aspartate; E, glutamate; F, phenylalanine; H, histidine; L, leucine; M, methionine; R, arginine.

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